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(54) Title: WOUND DRESSINGS AND PROCESSES FOR MANUFACTURE THEREOF (57) Abstract <p>A wound dressing comprises a sterile cell-growth supporting substrate comprising a crosslinked collagen conveniently in the form of a crosslinked collagen film disposed as part of a bilayer product upon a porous crosslinked or non-crosslinked collagen base. The collagen substrate carries epithelial cells, fibroblast cells or endothelial cells seeded on the substrate for example in the form of tissue fragments. The skin cells constitute preferably at least a sub-confluence cultured on a substrate and where a porous collagen is included in the substrate the substrate may contain an interstitial culture medium possibly in combination with interstitial growth hormone.</p>		

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WOUND DRESSINGS AND PROCESSES FOR MANUFACTURE THEREOF

This invention relates to skin cell-carrying wound dressings suitable particularly but not exclusively for the treatment of wounds where the epidermal and at least part of the dermal components have been lost. Such wounds include, for example, burns, leg ulcers, pressure sores and skin graft donor sites.

Skin cells are incapable of proliferating in vitro in suspension in a liquid culture medium. However, they can be made to proliferate in vitro on the surface of a suitable substrate and, under appropriate conditions, will multiply in stratified colonies and eventually produce a confluent layer. The substrate may, for example, be a synthetic polymer or collagen. Cultured skin cells may adhere to a polystyrene surface of a culture flask or other culture vessel.

The transfer of a patient of cultured skin cells grown in the above manner poses many difficulties. For example, although successful removal of a cultured skin cell layer from a culture flask can be accomplished with the assistance of enzymes, removal operations require great care and in any event leave a highly fragile product which is difficult to handle in both laboratory and clinical environments. However, cell cultures of this type have been used to investigate skin growth and have been used

clinically as skin grafts.

D Asselineau and M Prunieras, Brit J Dermatology (1984) III, Supplement 27, 219 - 222 discloses the in vitro seeding of epidermal cells on a lattice of fibroblasts cultured on non-crosslinked collagen. The need for a lattice which can be handled easily is recognized and addressed. Calf skin collagen in an amount of 4.5mg is mixed with 3×10^5 GM10 human embryonic skin fibroblasts in a culture medium based on Eagles MEM supplemented with 10% foetal calf serum to produce the lattice. However, the lattice has a vertical traction rupture strength of only 5 to 6g.

J F Burke et al, Ann Surg 94, 413, 1981 discloses the clinical use of a collagen-chondroitin 6-sulfate complex crosslinked with glutaraldehyde to form a sterilizable artificial dermis in the form of a gel. This artificial dermis is used in combination with a silastic artificial epidermis. The artificial dermis is grafted on to a wound bed and covered with the artificial epidermis, the former serving as a template for synthesis of fresh connective tissue. The artificial dermis gradually becomes host to populations of fibroblasts proliferating in a fresh connective tissue matrix which invades the artificial dermis, and vascularization takes place. The artificial dermis eventually suffers bio-absorption whereas the protective artificial epidermis remains intact and can be

removed when clinically appropriate to expose a developed neodermis. Epithelial coverage of the neodermis takes place subsequently by providing an epidermal autograft in place of the removed artificial epidermis.

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E Bell et al, Science, Vol 211, 6 March 1981, 1052 -1056 discloses in vitro seeding with autologous murine epidermal cells of a pre-graft consisting of autologous fibroblasts obtained from the same murine donor and cast in a non-crosslinked collagen lattice. The epidermal cells cover the lattice rapidly and differentiate producing a multi-layered artificial epidermis which keratinizes. The resulting grafts were autografted to the donor and generally found to have a good take and to undergo vascularization.

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B E Hull et al, A Clinical Trial of Biolayered Skin Equivalents, Surgery, Vol 107, No 5, 496 - 502 discloses the combining of allogenic fibroblast with non-crosslinked collagen. The resulting matrix was covered with a layer of autologous epidermal cells and cultured in vitro. It was found that epidermal growth was improved on the fibroblast-collagen matrix relative to in vitro growth previously experienced on non-crosslinked collagen per se. As expected, in murine grafting the resulting allograft did not become a target for immune rejection and in this respect performed comparably with grafts made of non-crosslinked collagen matrices populated only with allogenic

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fibroblasts. Similar findings are described in E Bell et al, Journal of Investigative Dermatology, 81:25 - 105, 1983 where epidermal tissue develops in vitro from keratinocytes plated on a dermal equivalent composed of fibroblasts in a non-crosslinked collagen matrix.

E Bell et al, Proc Natl Acad Sci USA, Vol 76, No 3, March 1979, 1274 - 1278 discloses very substantial contraction of a hydrated non-crosslinked collagen lattice as fibroblast cells grow and proliferate in vitro using the lattice as a substrate. Reductions in substrate area to about 3% of natural area are reported and are compared in principle to the contractive effects in vivo of cell interactions with physiological collagen and other fibrous physiological proteins during wound healing.

The culture of skin cells on collagen substrates is widely documented elsewhere and has been the subject of very considerable research effort. Non-crosslinked collagen substrate culture of skin cells is, for example, also described in A Eldad et al, Burns 13, 173 -180 (1987) and H Green et al, Proc Natl Acad Sc, 76, 5665 - 5668 (1979).

In addition to the handling difficulties associated with non-crosslinked collagen grafts, it has become increasingly clear that the take rate of cultured skin cells post-grafting is poor. This is reported, for example, in R Philip and B Gilchrest, J Derm Surg I (1989), 15, 1169.

This may partly be a result of wound bed inadequacy, infection of the wound bed and indeed to damage to the cell layers in the draft when it is transferred because of the poor mechanical strength of the graft.

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It will be recognized from the above review of literature that many recent techniques have concentrated upon the provision of a pseudodermis of fibroblast-collagen lattice upon which epithelial cells are cultured to form a bilayer.

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This pseudodermis is comprised on a non-crosslinked collagen gel seeded with fibroblast skin cells and cast in a polystyrene culture dish. The fibroblast cells cause the

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collagen gel to remodel and contract producing a structure that can more readily be handled. The fibroblast cells are

believed to improve epithelial migration and growth on the surface of the collagen gel substrate. This has been

reported in Hull et al, supra, and in Coulomb et al, J Invest Derm 92, 122 1989. These skin equivalents, apart

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from their relative durability and handling

characteristics, promote wound healing by providing a natural substrate for granulation tissue to form and for angiogenesis to occur. Allogenic fibroblast seeding

enables non-crosslinked collagen-fibroblast lattices to be prepared in advance. Autologous epidermal post-seeding

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produces an allograft not subject to immune rejection.

There are, however, several disadvantages in using a non-crosslinked collagen gel as a substrate. Non-crosslinked

collagen gels cannot be sterilized easily and this makes difficult the storage essential for keeping collagen substrate material readily available for use as a substrate and for keeping fibroblast-collagen lattices available for speedy epidermal post-culture. There are particular risks associated with storage of allograft lattices because of the possibility of transmission of infection from the donor. In use, it is necessary to rely on antibiotics and aseptic techniques to reduce the risk of infection and this is less reliable, more troublesome and more costly than sterilization. A further disadvantage of non-crosslinked collagen cells is that they may continue to contract under the influence of fibroblasts when applied to the patient as a graft. This can result in an increased risk of hypertrophic scarring, a clearly undesirable occurrence. Perhaps more importantly, it is usually necessary to allow epithelial cell layers to become fully confluent before graft transfer to the patient. Confluence of epithelium in vitro is usually reached in 14 to 21 days. This is often too long a delay in grafting treatment in the case, for example, of patients suffering from burns calling for urgent closure. This has tended to mean that autografting is precluded in practice in many cases. Whilst allografting combined with immunosuppressive therapy is available as a substitute, this is not always viable and, as mentioned above, is associated with risks of cross-infection (particularly viral cross-infection) between the graft donor and patient as well as possible

immunosuppressor side effects.

According to the invention there is provided in a first aspect a wound dressing which comprises a sterile cell-growth supporting substrate comprising crosslinked collagen and carrying skin cells.

The crosslinked collagen may be substantially anhydrous and whilst it may be porous so as to have a sponge-like constitution, this is by no means strictly essential.

The dressing may comprise either or both of epidermal cells and dermal cells but the invention has special application as a cultured epidermal cell carrier. The dressing may, for example, comprise epithelial cells such as keratinocytes, fibroblast cells or endothelial cells. A preferred dressing for clinical use comprises cultured fibroblast cells interstitially invasive of the collagen and a cultured layer of epithelium which has attained at least sub-confluence, the culture of two cell types being advantageous in that for clinical use, it is preferred to use cultivated autologous epithelial cells since an autologous cell culture produces little or no immunological rejection from the host (patient). It has been found surprisingly that it is not necessary to ensure an epithelial cell layer has reached confluence on the crosslinked collagen substrate before transferring the wound dressing on to the wound bed will have an acceptable

likelihood of taking. Conveniently, however, the cells should have reached at least 30% confluence before transfer, preferably at least 40% confluence with a confluence of 50% or more being most preferred. It has
5 been found that it takes from 5 to 7 days for epithelium to reach 50% confluence assuming an initial epithelial cell seeding density of 1.25×10^5 cells per cm^2 . Full confluence can be achieved in a period of from 7 to 10 days but a seeding density minimum of 6.25×10^4 cells per cm^2 is
10 a pre-requisite for full confluence using the method of Roly 1975. Crosslinked collagen substrates produced as described hereinbefore will support the growth of all types of cells normally found in mammalian dermis and epidermis, and specifically provides a much improved culture
15 environment for fibroblasts, and keratinocytes and other epithelial cells, as well as endothelial cells. Fibroblasts migrate through and divide within porous crosslinked collagen matrix and establish colonies faster than in non-crosslinked collagen and remodel the substrate
20 without noticeable shrinkage of the dressing.

In a particular embodiment of the invention, there is provided a wound dressing in the form of an allograft or autograft precursor dedicated to a specific wounded patient
25 and comprises of a porous crosslinked collagen matrix having allogenic or autologous fibroblast cells cultured interstitially therein and optionally a surface deposition of autologous epithelium or a surface sub-confluence of

autologous cultured epithelium.

The crosslinked collagen substrate will generally be in conformable sheet form. The term "sheet" is used as a convenient term of expression to denote a body having generally flat parallel opposed surfaces relatively large in size as compared to its thickness and includes collagen substrate in strip form.

The substrate may be produced from any commercially available natural collagen, for example bovine tendon collagen. In general, the collagen starting material is converted to an enzymatically digested uniform gel-like aqueous dispersion which can subsequently be cast, dried and covalently crosslinked. Suitable enzymes for digestion of the collagen include pepsin. Enzymatic digestion of the collagen is conducted in the presence of water and the resulting dispersion is generally homogenised and filtered to give a pourable liquid. The filter pore size may, for example, be less than $200\mu\text{m}$. Conveniently, the pore size of the filter will be less than $150\mu\text{m}$ and preferably less than $120\mu\text{m}$. Conveniently, however, the filter size will be greater than $70\mu\text{m}$ and preferably greater than $90\mu\text{m}$, for example $100\mu\text{m}$.

The concentration of collagen in the collagen dispersion is normally selected as a balance between the properties of the final product and the practicalities of the casting by

which the dispersion is converted to useful physical form. The concentration of collagen in the collagen gel dispersion will conveniently exceed 0.1% by weight. Concentrations greater than 0.25% by weight are preferred, particularly collagen gel concentrations of more than 0.5% by weight. The collagen concentration may preferably be about 2% by weight although this figure may be slightly exceeded in certain cases.

Conveniently, the collagen dispersion is cast to form a sheet.

The thickness of the cast sheet of collagen will depend on the depth of wound intended to be filled by the final dressing, subject generally to limitations imposed by practical needs for conformability. The thickness of the cast collagen sheet will generally not be greater than 20mm or less than 0.5mm, typically from 1 to 10mm. In practical terms, it should be thick enough to handle and to carry a culture of skin cells and thin enough for conformability. The thickness of the collagen will not in practice unacceptably restrict nutrients from reaching the cells carried by the crosslinked collagen substrate especially when the substrate is in porous form. Substantial final dressing thicknesses are viable in practice and enable the building up of a wound bed in deep wounds so as to restore skin contour.

Drying of the cast collagen sheet is in practice often effected prior to crosslinking in the interests of ease of effective crosslinking, freeze-drying being preferred, but crosslinking can be effected pre-drying.

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The collagen in the so-cast sheet can conveniently be crosslinked using glutaraldehyde or formaldehyde although other crosslinkers, eg diamines, isocyanates and dicarboxylic acids can be used. Formaldehyde is preferred.

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A typical crosslinking process comprises vapour tanning the cast collagen sheet with formaldehyde.

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Pre-processed commercially available crosslinked collagen suitable for use in the invention is sold under the trade mark COLLASTAT.

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A similar crosslinked collagen is one which has been renatured and covalently crosslinked utilizing readily available polyfunctional crosslinking agents, such as dialdehydes, dicarboxylic acids and diamines in a procedure which involves dissolving tropo-collagen in a buffer of pH 3.0 to 5.0 wherein the solution contains approximately 1% to 2% by weight of the collagen. Then 1% of a dialdehyde crosslinking agent such as glutaraldehyde or formaldehyde is added and the mixture frozen and stored for approximately 24 hours.

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Preferred substrates for use in the invention are of collagen crosslinked to a shrink temperature in the range from 40°C to 85°C (typically 55°C to 65°C).

5 In any event, post-crosslinking the collagen sheet is generally cut to a suitable size, packaged in bacteria proof pouches and terminally sterilized. The preferred method of sterilization is gamma irradiation practised after pouch sealing but as an alternative ethylene oxide
10 gas may be brought into contact with the crosslinked collagen prior to sealing and residual amounts of ethylene oxide may be included in the pouch.

Residual crosslinking agent which may be present after the
15 crosslinking process may have a cytotoxic effect on the use of the crosslinked collagen as a substrate to support skin cell proliferation and must be removed to the point where the substrate is viable for this purpose. Removal will usually be carried out by washing, a sterile phosphate-
20 buffered saline wash being preferred. Desirably, a formaldehyde residue should not exceed 0.02mg/g since levels above this are likely to be skin cell cytotoxic.

Collagen pore size in known uses of non-crosslinked
25 collagen for wound dressing is variable and has an influence on, for example, fibroblast proliferation and substrate invasion as well as angiogenesis. Whilst porosity is not an essential characteristic for the

substrates used in this invention, it is advantageous for culture of fibroblast cells and accordingly dressings based on porous substrates represent a preferred embodiment of the invention. In practice, porosity characteristics in substrates used in this invention will be selected so that fibroblast migration into the body of the substrate is accommodated and for this purpose pores $10\mu\text{m}$ and above in size are desirable. The minimum pore size in a substrate will conveniently be $20\mu\text{m}$ or more, for example, a pore size minimum greater than $30\mu\text{m}$ (eg $50\mu\text{m}$) being a likely preference in practice. The range of pore sizes in the substrates used according to the invention will generally have a ceiling of less than $1000\mu\text{m}$. A dressing with a pore size ceiling of $300\mu\text{m}$ is effective. An example of a suitable maximum pore size is $175\mu\text{m}$ or less (eg $150\mu\text{m}$). Pore size in preferred embodiments will thus range from $20\mu\text{m}$ to $300\mu\text{m}$, for example, 20 to $250\mu\text{m}$ or 50 to $300\mu\text{m}$.

The crosslinked collagen may have a porous structure of which the mean pore size is in the range from $50\mu\text{m}$ to $150\mu\text{m}$, the pores conveniently having a size distribution of from $20\mu\text{m}$ to $250\mu\text{m}$.

Porous substrates used in this invention can contain liquid culture medium and growth hormone interstitially.

Skin cells may be seeded on to the substrates used according to the invention.

Epithelial cells in dressings according to the invention are generally grown on the collagen substrate with the substrate immersed in a liquid culture medium to provide a substrate surface at the air:liquid interface. The cells then grow, stratify and differentiate at the interface. In cast substrates, the surface on which the substrate is cast in production creates a smoother surface for growth of cells. It is accordingly this surface which should preferably be exposed at the air:liquid interface.

The substrate may conveniently be in the form of a laminated bi-layer with such a surface exposed for receipt of a deposition of cells to be grown.

Beneficial improvement in surface smoothness can be accomplished by lamination of a crosslinked collagen film first layer for carrying skin cells (eg one cast from a collagen dispersion containing 5% by weight or more of collagen, preferably 5% to 8% by weight of collagen) to a second collagen layer (eg a collagen sponge) which may be crosslinked or non-crosslinked, typically to the surface thereof on which the substrate second layer was cast. Although, as just indicated, the second layer may be crosslinked, in practice it may be preferred that the second layer will not be crosslinked; where the second layer is crosslinked, the film collagen may conveniently be crosslinked to substantially the same degree as the

collagen in the second layer. In practice, crosslinked collagen sponges used in the present invention need only be lightly crosslinked. The film first layer may conveniently be substantially non-porous.

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Surface smoothness is less important for culture of fibroblast cells. Fibroblast cells migrate into a porous crosslinked collagen substrate even when deposited only at the substrate surface. However, fibroblast cells for culture are usually carried into the body of porous collagen by liquid culture medium and proliferate therein.

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Of course, tissue fragments such as skin plugs can be used to provide cells for culturing, the substrate conveniently being provided with receptacles therein for reception of such tissue fragments. Autologous skin plugs from the host (patient) can be excised and placed into corresponding holes in the collagen matrix. Such plugs would typically be 2 to 3mm in diameter. This provides for an immediately transferable dressing which could be used with immediacy for application over leg ulcers, traumatic excisions, and pressure sores. The skin cells migrate from the skin plugs into and on to the collagen matrix in situ and the matrix is subsequently resorbed into the wound. Whilst this provides immediacy of a treatment which will in due course close a wound by autografting, it has inadequate efficacy in the case of severe extensive burn injuries where speed of in situ confluence over an area between relatively

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distant wound margins is required for acceptable wound closure and perhaps patient survival.

5 It is because allografting is subject to immune rejection and the culture time to produce an epithelial autograft to transfer confluence using other substrates known in the art is generally measured in periods of 14 to 21 days from patient admittance that the dressings according to the invention are so surprisingly significant. Whereas an
10 allograft would need to be available from pre-production store and an autograft would be either unavailable, available only for late treatment or available quickly only for planned surgery (eg in the case, for example, of plastic surgery), the invention enables a graft which has
15 achieved transfer confluence to be available in a short time, perhaps only 5 days, without the need for prior planning or storage.

20 In all embodiments of the invention the wound dressings described can be resorbed after 14 days with likely replacement by the patient's own skin tissue. A particular feature of the present invention is that because the collagen matrix does not contract significantly when placed into a wound, it reduces the risk of hypertrophic scar
25 development.

Crosslinked collagen substrate can be sterilized easily making storage and use less difficult. When seeded with

fibroblasts in culture, the substrate does not contract significantly. This means that the dressing is substantially the same size at the transfer stage as it is at the cell seeding stage giving practical advantages in use.

In a second aspect, the invention provides a skin graft kit comprising a package housing a sterile crosslinked collagen sheet serving as a substrate for receipt in use of the kit of a deposit of epithelial cells and a sterile culture vessel sized to contain the collagen sheet, the collagen sheet and the culture vessel being disposed in a sterile environment defined by a bacterially-impermeable membrane. The package may provide a wall defining the bacterially-impermeable membrane. Alternatively, of course, the package houses a bacterially-impermeable membrane envelope containing said collagen sheet and said culture vessel together or two such envelopes containing said collagen sheet and said culture vessel separately. The collagen sheet may support pre-cultured fibroblasts and be frozen.

The invention also includes within its scope a method, whose preferred features are mentioned hereinbefore, of producing a crosslinked collagen skin cell culture substrate which method comprises treating collagen sheet crosslinked with a crosslinking agent which is incidentally cytotoxic to mammalian skin cells to remove crosslinking agent unconsumed by the crosslinking reaction, the

treatment being continued until the sheet is capable of supporting skin cell culture as signified by survival of 80% of a deposit of skin cells on the substrate 12 hours after deposition.

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The invention includes within its scope an internally implantable bioabsorbable wound healing device comprising a sterile cell growth supporting substrate comprising crosslinked collagen and carrying endothelial or other tissue cells for in situ growth to supply endothelial or other tissue at the site of an internal wound.

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The invention also includes a method of wound treatment which method comprises applying to the wound (eg a peripheral wound) a wound dressing according to the invention of applying internally to a wound an implantable device as just described. As mentioned earlier, the wound if external may be a burn, leg ulcer or skin graft donor site, or it may be a bed sore or other pressure sore. The dressing is useful for both full thickness and partial thickness skin wounds and for deep wounds may be of sufficient thickness to pack out the wound so as to restore skin profile.

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For cosmetic purposes, the invention provides a method of cosmetic skin treatment applied topically to an animal body skin surface which has suffered surgical or non-surgical perforative trauma which method comprises dressing to said

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surface a wound dressing according to the invention.

Included within the scope of the invention is use of crosslinked collagen as a substrate for in vitro culture of skin cells to produce a live tissue graft comprising skin cells and a crosslinked collagen substrate to which said skin cells are layer is adhered.

The following specific Examples illustrate the invention.

In the photomicrographs representing the drawings:-

Figure 1 is a photomicrograph X 20 obj showing the absence of outgrowth of epithelium from a skin plug placed in Collastat after 12 days;

Figure 2 is a photomicrograph X 10 obj showing neutral red staining of epidermal cells cultured on Collastat for 13 days;

Figure 3 shows a histological section (H & E; X 10 obj) through Collastat seeded with opidermal cells and cultured for 13 days; and

Figure 4 is a photomicrograph showing indirect immunoperoxidase staining of a dispased sheet of cultured epithelium grown on the top of a Collastat dressing for 4 days.

Example 1

Epithelium was cultured from skin plugs. A series of juvenile foreskin skin plugs 2 to 3mm in diameter were excised from human foreskin tissue discarded during surgery. Holes to accommodate the skin plugs were punctured in Collastat sheets and the plugs placed in the holes. The resulting cultures were each fed basally using the method of Barlow & Pye described in Barlow Y and Pye R J, Keratinocyte Culture in Methods in Molecular Biology, 1990, Ch 9, Humana Press.

After 12 days, histological investigation showed no outgrowth of epithelium from the skin plugs, as shown in Figure 1 (in Figure 1, a. is the epidermis of the skin plug, b. is the dermis and c. is the Collastat matrix). It is very difficult accurately to position juvenile foreskin because of its pliable nature and for effective growth of epithelial cells the skin surface should be flush with the Collastat. The lack of outgrowth may also be the result of poor diffusion of culture nutrients through the whole thickness of the skin plugs and this conjecture is somewhat supported by degeneration of tissue found in histological examination of the skin plugs.

Example 2

Epithelium was cultured by seeding of epithelial cells on

Collastat. Epidermal cells isolated from human foreskin tissue discarded during surgery were seeded at seeding densities of, respectively, 1.5×10^6 cells and 3.6×10^6 cells on to two 25mm x 25mm Collagen sheet dressings.

5 Adopting basal feeding using the keratinocyte culture medium referred to in Example 1, the seeded cells were cultured following the methods set out in Barlow Y and Pye RJ, Keratinocyte Culture in Methods in Molecular Biology, 1990, Ch 9, Humana Press. Histological examination reveals
10 substantial epithelial cell growth after 20 days in the case of the culture seeded at a density of 1.5×10^6 cells. Broadly equivalent epithelial development was observed in the higher seeding density culture after 13 days, with surface spread of epithelial cells, as shown in Figure 2
15 (in Figure 2, a. indicates epidermal cells and b. indicates collagen matrix), and some migration of epithelium into the body of the Collastat, as represented by Figure 3 (in Figure 3, a. indicates epidermal cells and b. indicates collagen matrix).

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Example 3

Epithelial confluence was cultured on Collastat. Epidermal cells (approximately 5.25×10^4 cells cm^{-2}) were cultured to
25 confluence in a culture flask according to the method of Barlow & Pye supra and the resulting sheet epidermis dispased from the culture flask and transferred without inversion on to a Collastat sheet. The resulting culture

was incubated for four days basally fed with the culture medium described in Example 1. Epithelial development was examined post-incubation, the results being shown in Figure 4 (in Figure 4, a. indicates cultured epithelium growing on the top of the Collastat dressing, b. indicates migration of epithelium into the porous structure along the collagen and c. indicates collagen matrix).

Example 4

Fibroblasts and endothelial cells were grown interstitially in a Collagen matrix. Human dermal fibroblasts and bovine endothelial cells were seeded at a concentration of 4×10^4 cells cm^{-2} on to Collastat sheet dressings pre-hydrated with 1 to 2 ml of a culture medium comprising DMEM supplemented with 10% by weight foetal calf serum. The cultures were incubated for up to 11 days at 37°C . Selected dressings were pulse labelled with tritiated thymidine ($^3\text{HTdR}$) at various points in time ($^3\text{HTdR}$ is utilized by cells synthesizing DNA and is therefore an indicator of cell growth). Assessment of cellular incorporation of $^3\text{HTdR}$ was carried out by incubating the selected cultures with $^3\text{HTdR}$ for 4 hours. Each so-treated dressing was then rinsed several times on a sintered glass filter (porosity G3) in ice cold phosphate buffered saline (PBS) under gentle vacuum. The dressing was then fixed in 10% trichloroacetic acid (TCA) and the radioactivity in TCA precipitated material solubilized in 1% SDS was measured, followed where

necessary by complete solubilization of the collagen dressing in Optisolv.

Over the 11 day period, a linear increase in the
5 incorporation of $^3\text{HTdR}$ by cells was observed.

In addition, Collastat dressings containing cells were stained at selected intervals with neutral red vital stain. Viability was assessed by incorporation of dye into the
10 cells and by their morphology. Cells were observed to migrate through the entire dressing over a period of 52 days and to divide within the dressing material.

Example 5

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Fibroblasts isolated from explant culture of human skin were seeded into Collastat dressings containing 0.02 mg maximum of formaldehyde per gram at a concentration of 4×10^4 cells cm^{-2} and were cultured in DMEM + 10% foetal calf
20 serum for 2 to 3 days at 37°C . The dressings containing the so cultured fibroblasts were then raised to an air-liquid interface. The Collostat-fibroblast dressings were seeded with epithelial cells from the same donor, isolated by the method of Barlow & Pye supra at a density of 1.25×10^5
25 cells cm^{-2} and allowed to grow for 7 to 14 days at 37°C until confluence was reached. The cultures were fed basally during this period in 3DMEM:F12 containing growth factor supplements and 10% by weight foetal calf serum (Rheinwald

& Green, 1975). It was found that cultures produced in this way could be transplanted to wounds after 4 days at which time the epithelial cells had not achieved confluence, as an autograft which over the course of several weeks became bio-absorbed by the patient after closing the wounds.

Example 6

Epithelium was grown as an intact sheet over a period of 10 days on tissue culture plastic adopting the method of Barlow & Pye supra. The epithelial sheet was enzymatically removed by dispase. The sheet was then transferred to the top of a Collostaf-fibroblast intermediate dressing as described in Example 5 and basally fed as described in Example 5. The epithelial sheet was maintained at an air-liquid interface and further cultured for about 4 days in vitro to allow the epithelium to adhere well to the collagen. Autografting of the resulting dressing was then effected as described in Example 5 with comparable results.

Example 7

Composite substrates were prepared in the following manner:-

A collagen dispersion containing 8% by weight collagen was cast to a thickness of 2mm on to sheets of siliconised

paper and further sheets of siliconised paper placed over the exposed film surfaces. The laminates were then allowed to stand overnight at room temperature to partially dry. One of the release sheets was removed from each sample and the exposed surface contacted with the surface of a crosslinked collagen sheet. The crosslinked collagen sheets contained either 0.75% or 2.0% collagen by weight. The remaining release sheets were removed and the composites allowed to stand for a further overnight period at room temperature to fully dry the film component. Upon drying, the total thickness of the composite was between 3 and 4mm and the thickness of the film component was less than 0.5mm. After drying, the composites were sterilized by either an ethylene oxide sterilization procedure or by gamma irradiation.

The composite collagen substrates were rinsed in phosphate buffered saline prior to culture of epithelial cells. The composites were then pre-hydrated with tissue culture medium as described in Example 2. The keratinocytes were seeded on to the surface of the films at seeding densities of approximately 1.25×10^5 cells per cm^2 . After 24 hours keratinocytes were observed to attach to the collagen films. Neutral red vital staining of these cells indicated that cells continued to divide and grow on the surface of the collagen film but did not migrate into the porous collagen sheet. After 72 hours, keratinocytes were observed to have achieved approximately 50% confluence, and

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full confluence was achieved after a period of between a week and ten days.

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Claims

1. A wound dressing which comprises a sterile cell-growth supporting substrate comprising crosslinked collagen and carrying skin cells.

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2. A wound dressing as claimed in Claim 1 wherein the substrate comprises collagen crosslinked to a shrink temperature in the range from 40° to 85°C.

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3. A wound dressing as claimed in Claim 1 or Claim 2 wherein the crosslinked collagen has a porous structure of which the mean pore size is in the range from 50 μ m to 150 μ m, the pores having a size distribution of from 20 μ m to 250 μ m.

15

4. A wound dressing as claimed in any one of Claims 1 to 3 wherein the substrate comprises a first layer of a crosslinked collagen and a second layer of a porous collagen.

20

5. A wound dressing as claimed in Claim 4 wherein the second layer comprises a non-crosslinked collagen.

25

6. A wound dressing as claimed in any preceding claim wherein the substrate has a thickness of from 1mm to 10mm.

7. A wound dressing as claimed in any preceding claim

wherein the substrate is porous and contains interstitial culture medium.

8. A wound dressing as claimed in any preceding claim
5 wherein the substrate is porous and contains interstitial growth hormone.

9. A wound dressing as claimed in any preceding claim
10 wherein the skin cells comprise epidermal cells and/or dermal cells.

10. A wound dressing as claimed in any preceding claim
wherein the skin cells comprise epithelial cells, fibroblast cells or endothelial cells.

15 11. A wound dressing as claimed in Claim 10 wherein the epithelial cells are keratinocytes.

12. A wound dressing as claimed in any preceding claim
20 wherein the skin cells comprise cultured seeded cells.

13. A wound dressing as claimed in any preceding claim
wherein the skin cells comprise cells in the form of tissue fragments.

25 14. A wound dressing as claimed in Claim 13 wherein the substrate is provided with receptacles therein and said tissue fragments are received in said receptacles.

15. A wound dressing as claimed in Claim 13 or Claim 14 wherein said tissue fragments are skin plugs.

5 16. A wound dressing as claimed in any preceding claim wherein the skin cells constitute a sub-confluence cultured on said substrate.

10 17. A wound dressing as claimed in any preceding claim and in the form of an allograft or autograft precursor dedicated to a specific wounded patient and comprised of a porous crosslinked collagen matrix having allogenic or autologous fibroblast cells cultured interstitially therein.

15

18. A wound dressing as claimed in Claim 17 wherein the fibroblastic crosslinked collagen matrix has a surface deposition of autologous epithelium.

20 19. A wound dressing as claimed in Claim 17 wherein the fibroblastic collagen matrix has a surface sub-confluence of autologous epithelium cultured thereon.

25 20. A wound dressing substantially as hereinbefore described in any one of the foregoing specific Examples.

21. A skin graft kit comprising (i) a package housing a collagen substrate for receipt in use of the kit of a

deposit of epithelial cells and comprising a sterile crosslinked collagen sheet cell carrier and (ii) a sterile culture vessel sized to contain the collagen substrate, the collagen substrate and the culture vessel being disposed in
5 a sterile environment defined by a bacterially-impermeable membrane.

22. A package as claimed in Claim 21 wherein the collagen sheet supports pre-cultured fibroblasts and the collagen
10 substrate is frozen.

23. A kit as claimed in Claim 21 or Claim 22 wherein the package provides a wall defining the bacterially-impermeable membrane.
15

24. A kit as claimed in Claim 21 or Claim 22 wherein the package houses a bacterially-impermeable membrane envelope containing said collagen substrate and said culture vessel together or two such envelopes containing said collagen
20 substrate and said culture vessel separately.

25. A method of wound treatment, the method comprising dressing to a wound a wound dressing as claimed in any one of Claims 1 to 20.

25.

26. A method as claimed in Claim 25 wherein said wound is a partial thickness skin wound.

27. A method as claimed in Claim 25 wherein said wound is a full thickness skin wound.

28. A method as claimed in any one of Claims 25 to 27
5 wherein said wound is a burn.

29. A method as claimed in any one of Claims 25 to 27 wherein said wound is a leg ulcer.

10 30. A method as claimed in any one of Claims 25 to 27 wherein said wound is a pressure sore.

31. A method as claimed in Claim 30 wherein said pressure sore is a bed sore.

15 32. A method as claimed in any one of Claims 25 to 27 wherein said wound is a skin graft donor site.

20 33. A method of cosmetic skin treatment applied topically to an animal body skin surface which has suffered surgical or non-surgical perforative trauma which method comprises dressing to said surface a wound dressing as claimed in any one of Claims 1 to 20.

25 34. A method as claimed in Claim 33 wherein the perforative trauma has full thickness skin perforation.

35. Use of crosslinked collagen as a substrate for in

vitro culture of skin cells to produce a live tissue graft comprising skin cells and a substrate comprising crosslinked collagen to which substrate said skin cells are adhered.

5

36. A method of producing a crosslinked collagen skin cell culture substrate which method comprises treating collagen sheet crosslinked with a crosslinking agent which is incidentally cytotoxic to mammalian skin cells to remove crosslinking agent unconsumed by the crosslinking reaction, the treatment being continued until the sheet is capable of supporting skin cell culture as signified by survival 12 hours after deposition of 80% of a deposit of skin cells on the substrate.

15

37. A method as claimed in Claim 36 wherein the crosslinked collagen sheet is treated by washing with a solvent for the crosslinking agent.

20

38. A method as claimed in Claim 36 or Claim 37 wherein the crosslinked collagen sheet is treated until the content of unconsumed crosslinking agent is not more than 0.02mg crosslinking agent per gram of the crosslinked collagen sheet.

25

39. A method as claimed in any one of claims 36 to 38 wherein the crosslinked collagen has been crosslinked with formaldehyde, glutaraldehyde or another water-soluble

crosslinking agent.

40. A method as claimed in Claim 39 wherein the
crosslinked collagen is treated to remove unconsumed
5 formaldehyde, glutaraldehyde or other crosslinking agent by
washing the sheet with phosphate-buffered saline wash.

41. A method of producing a crosslinked collagen skin
cell culture substrate, substantially as hereinbefore
10 described in any one of the foregoing specific Examples.

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FIG. 1

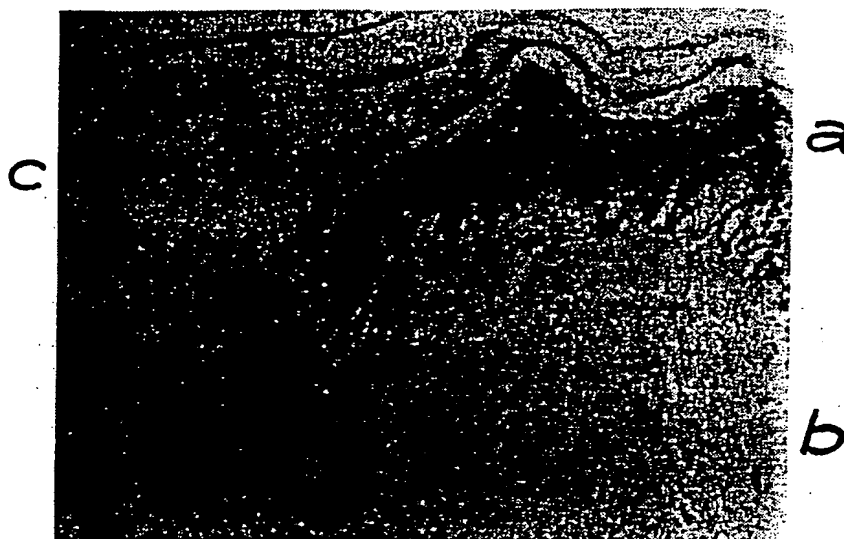
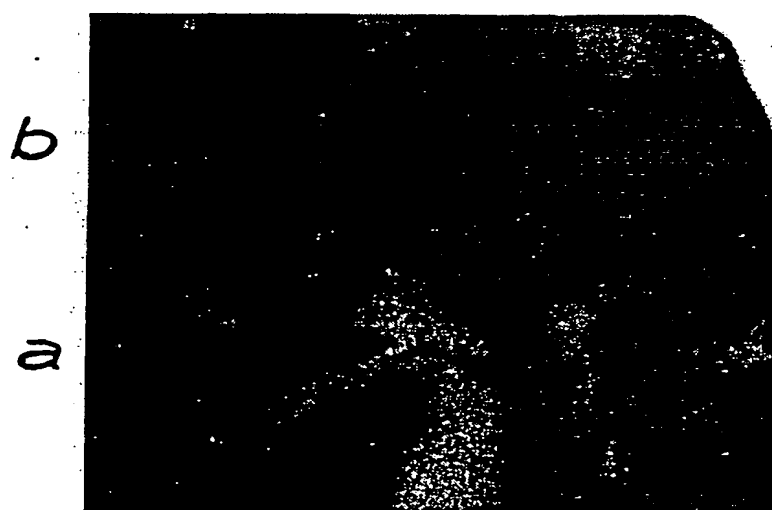


FIG. 2



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FIG.3

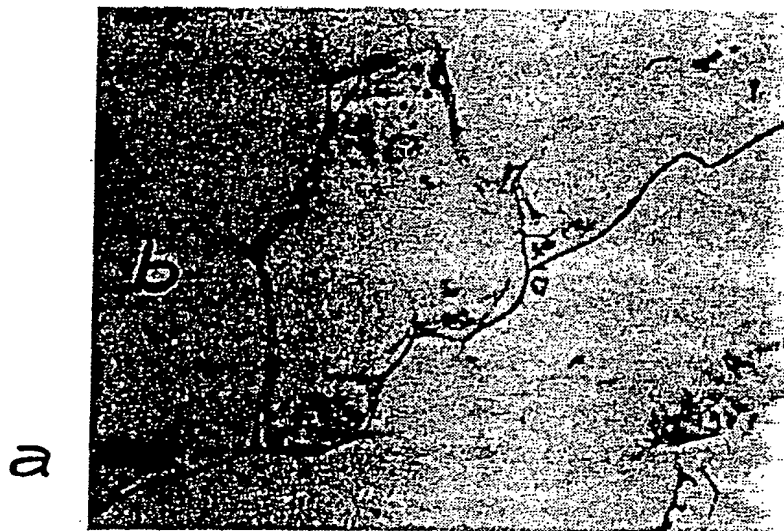
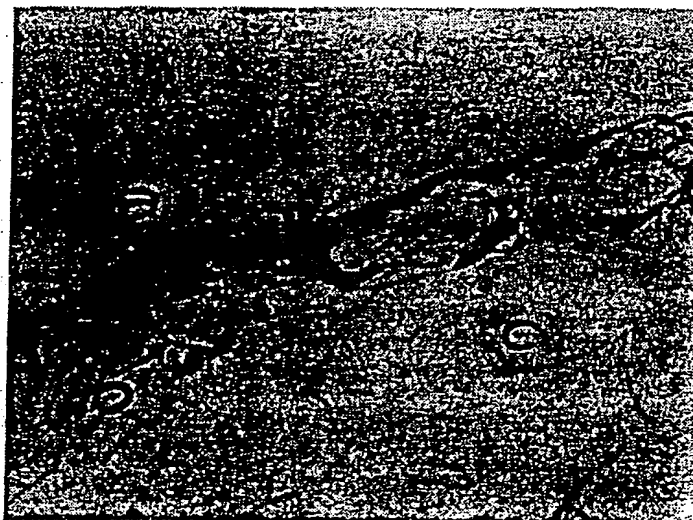


FIG.4



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International Application No.

PCT/GB 91/02146

Form PCT/ISA/210 (second sheet) January 1985

III DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Biotechnology and Applied Biochemistry, vol. 12, no. 1, February 1990, Academic Press Inc., K.B. HEY et al.: "Crosslinked fibrous collagen for use as a dermal implant: Control of the cytotoxic effects of glutaraldehyde and dimethylsuberimidate", pages 85-93, see paragraph: "Materials and methods" ---	1
X	EP,A,0243132 (KOKEN CO., LTD) 28 October 1987, see page 3; claims ---	1-19
X	Experimental Cell Research, vol. 191, no. 1, November 1990, Academic Press, Inc., J. TIOLLIER et al.: "Fibroblast behavior on gels of type I,III and IV human placental collagens", pages 95-104, see abstract ---	1
P,X	Experimental Cell Research, vol. 193, no. 2, April 1991, Academic Press, Inc., E. TINOIS et al.: "In vitro and post-transplantation differentiation of human keratinocytes grown on the human type IV collagen film of a bilayered dermal substitute", pages 310-319, see paragraph: "Discussion" -----	1-19

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

REMARK: ALTHOUGH CLAIMS 25-32 ARE DIRECTED TO A METHOD OF TREATMENT OF THE HUMAN OR ANIMAL BODY THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED EFFECTS OF THE COMPOUNDS.

2. ☐ Claim numbers 20, 41 because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

PLEASE SEE PCT RULE 6.2.

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple Inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9102146
SA 54066

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 15/05/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0396138	07-11-90	US-A- 4996154	26-02-91
		JP-A- 3164168	16-07-91
EP-A- 0243132	28-10-87	JP-A- 62246371	27-10-87
		US-A- 4883487	28-11-89

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82